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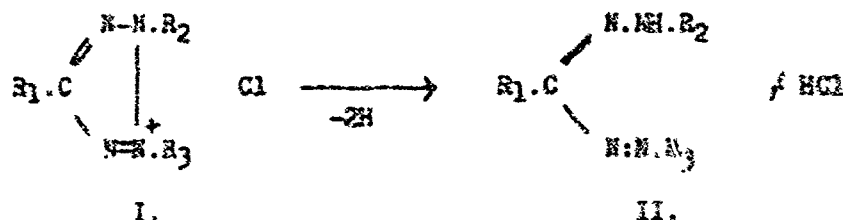
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The determination of the reduction potential of tetrazolium compounds.

by B. Jerchel and K. Moshle.

Ber. d. Deutschen Chem. Ges. 77: 591-601 (1944).

According to G. Lakon (1), 2,3,5-triphenyl-tetrazolium chloride, (I, $R_1 = R_2 = R_3 = C_6H_5$) is important as a stain indicator in the determination of the viability of cereal and corn seeds. The colorless tetrazolium salt is hydrogenated into dark red, poorly water-soluble, air-stable triphenyl formazan (II, $R_1 = R_2 = R_3 = C_6H_5$) by viable germinal tissue (2). It seemed promising, therefore, to investigate the reduction of tetrazolium compounds potentiometrically.



An attempt to establish a reduction-oxidation potential for the system tetrazolium salt/formazan in the usual manner (3), failed. A reductional process with an irreversible course is involved here. The experience gained in the preparative treatment of these compounds (4) had revealed the following: Tetrazolium salts may be reduced to formazans by numerous reductants under the most diversified conditions; the dehydration of these formazans to tetrazolium compounds, on the other hand, must be conducted under quite particular conditions (with lead tetraacetate, amyl nitrite or mercuric oxide as oxidizing agent).

The work of J.B. Conant (5) and L.F. Fieser (6) has established theoretical and practical prerequisites for the determination of the electromotive forces in irreversible oxidation-reduction processes.

The redox potential of an unknown compound may be determined by metric comparison of a known compound with the unknown, reversible compound. The mixture of the oxidized and reduced forms of reversible indicator systems under varied conditions yields a series of reductional systems with known potentials. An unknown substance in the amount equimolecular to the leuco form is added. After a few minutes the adjustment of constant potentials is observed. They indicate the new equilibrium state between known and unknown redox systems. If these values are represented graphically in relation to the corresponding indicator potentials, the resulting points may be connected by a straight

line. The point obtained by extrapolation to 0 volts deviation as point of intersection of the line with the ordinate, indicates the redox potential of the unknown substance. Fig. 1 and 2 represent this type of redox potential determination for safranin at pH 1.02 and for anthrachinon-disulfonate-(2.7) at pH 1.02 and 6.72.

It is remarkable how closely the values found by this indirect method agree with the redox potentials determined in the usual manner (Table 1).

TABLE 1.

pH	Substance	Normal potential (calomel- electrode)	Normal potential (determined in- directly)
		Volts	
1.02	Anthrachinon-disulfonate-(2.7)	-0.073	-0.077
	Safranin	-0.101	-0.101
6.72	Anthrachinon-disulfonate-(2.7)	-0.403	-0.401

Upon combination of different known, reversible redox systems with the irreversible test substance, potential changes are observed, however, which indicate the known redox systems between which the irreversible oxidation-reduction reaction is to be incorporated. J.B. Conant (5) coined the concept of "apparent oxidation potential (AOP)" or "apparent reduction potential (ARP)" for potential derived by this method — equating it for practical reasons with that of a redox system that is oxidized or reduced only to 20-30% within 30 minutes.

Departing from this potential concept, L.F. Fieser (6) later defined another related concept, that of "critical potential." Rates of reaction are compared in the determination of AOP or ARP. Since the constants, for example, of the rate of dehydration of different compounds may have different values, and non-comparable potentials would thus be measured, Fieser searches for reference redox systems which produce only small reactions in a short time. He measures potential changes caused by different systems within 5 minutes. By appropriate extrapolation he then obtains the values designated "critical oxidation potentials" (7).

The approximate position of the reduction potential of tetrazolium compounds was determined by means of different stain indicators for purposes of orientation (2). A clue is obtained thereby as to the indicator substance which is feasible as a comparative system in potential measurement.

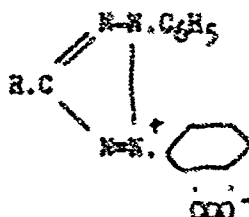
If the measurement of redox potential is conducted with the aid of the electrometric method, the process is the following: Systems with different potentials are produced by the mixture of reduced and oxidized forms of reversible indicators. To the mixture is added an amount of

tetrazolium salt (the acceptor substance) equimolecular to the reduced portion of the indicator. Now, if the adjusted potential of the indicator is more negative than the reduction potential of the acceptor, the former may be reduced. This is recognized by a distinct potential variation, measured with platinum electrodes against a calomel electrode according to the principle of the compensational method. The magnitude of the measured potential change depends on the existing difference between indicator potential and the reduction potential of the acceptor substance. The progression of potential change in relation to the time is depicted in fig. 3, using the reduction of triphenyl tetrazolium chloride as an example.

Unstable redox systems perform similarly. Their adjusting equilibrium is constantly disturbed by secondary reactions. The values measured and then expressed in percent of the used oxidizing agent, were inserted in an empirical formula by Fieser. In this manner he obtained so-called values of percentual oxidation which, entered against the matching indicator potential values, were located on a line. However, if this formula is used in conjunction with the measured results of tetrazolium salts, no satisfactory presentation can be achieved. Here the entry of potential changes occurring in the first 5 minutes, in logarithmic units, against the corresponding indicator potential values (in normal units) leads to points which may be connected by a line (Fig.3).

An extrapolation to 0 millivolts potential change is not feasible. For this reason a potential change of 1 millivolt within the first 5 minutes is empirically fixed as the standard value. The definition of this value at 1 mV corresponds best to the measured tolerance attainable with the apparatus. The importance of the measured values is due to the circumstance that they permit the exact comparison of the redox potential of different substances which change irreversibly upon reduction.

Table 2 reflects a tabulation of reduction potential values established for the various tetrazolium salts. At pH 6.72 the potentials of 2,3,5-triphenyl-, 2,3-diphenyl-5-carboxy-, 2,3-diphenyl-5-methyl-, 2-phenyl-3-(p-oxy-phenyl)- and 2-phenyl-3-(p-carboxy-phenyl)-5-methyl-tetrazolium chloride have almost exactly the same magnitude. 2-phenyl-3-(o-carboxy-phenyl)-5-methyl- and 2-phenyl-3-(o-carboxy-phenyl)-5-n-heptyl-tetrazolium chloride, on the other hand, are far more difficult to reduce. Their reduction potentials are more negative by about 140 mV. These compounds probably are in solution in the form of amphoteric ions (III). It may be assumed, therefore, that the negative charge of the carboxyl group, which is proximal to the positive charge of the tetrazol nucleus, has an influence on the redox potential (8).



III.

TABLE 2: Determination of the reduction potentials of different tetrasolium salts.

Acceptor substance	Indicator	Adjusted indicator potential mV	Change for 0-5 min mV	pH	Reduction potential (change 1 mV/0-5 min) calomel el. H ₂ el. Volts	
$\begin{array}{c} \text{C}_6\text{H}_5\text{.C} \begin{array}{l} \nearrow \text{N-N.C}_6\text{H}_5 \\ \searrow \text{N=N}^+ \end{array} \text{C}_6\text{H}_5 \\ \text{Cl}^- \end{array} \quad (9)$	Indigo-tri-sulfonate	301.8	0.1	6.72	-332	-0.083
		310.4	0.23			
		319.5	0.5			
		320.6	0.57			
		328.8	0.93			
		335.9	1.23			
		340.1	2.63			
		349.0	4.0			
	Indigo-di-sulfonate	336.7	1.06			
		336.8	1.4			
$\begin{array}{c} \text{HOOC.C} \begin{array}{l} \nearrow \text{N-N.C}_6\text{H}_5 \\ \searrow \text{N=N}^+ \end{array} \text{C}_6\text{H}_5 \\ \text{Cl}^- \end{array} \quad (10)$		344.1	2.3	6.72	-330	-0.081
		349.2	3.1			
		350.0	3.3			
		354.5	5.1			
		354.6	6.1			
		359.9	8.5			
		378.4	30.0			
	anthraquinon-di-sulfonate-(2.7)	333.7	0.77			
$\begin{array}{c} \text{CH}_3\text{.C} \begin{array}{l} \nearrow \text{N-N.C}_6\text{H}_5 \\ \searrow \text{N=N}^+ \end{array} \text{C}_6\text{H}_5 \\ \text{Cl}^- \end{array} \quad (11)$	Indigo-tri-sulfonate	325.2	0.7	6.72	-338	-0.089
		334.2	1.8			
		346.9	2.0			
$\begin{array}{c} \text{CH}_3\text{.C} \begin{array}{l} \nearrow \text{N-N.C}_6\text{H}_5 \\ \searrow \text{N=N}^+ \end{array} \text{C}_6\text{H}_5 \\ \text{Cl}^- \end{array} \quad (12)$	Indigo-tri-sulfonate	322.7	0.5	6.72	-337	-0.088
		336.0	0.8			
		348.1	2.0			
$\begin{array}{c} \text{CH}_3\text{.C} \begin{array}{l} \nearrow \text{N-N.C}_6\text{H}_5 \\ \searrow \text{N=N}^+ \end{array} \text{C}_6\text{H}_5 \\ \text{Cl}^- \end{array} \quad (13)$	Indigo-tri-sulfonate	323.2	0.7	6.72	-328	-0.079
		335.8	1.9			
		348.0	5.1			
$\begin{array}{c} \text{CH}_3\text{.C} \begin{array}{l} \nearrow \text{N-N.C}_6\text{H}_5 \\ \searrow \text{N=N}^+ \end{array} \text{C}_6\text{H}_5 \\ \text{Cl}^- \end{array} \quad (14)$	anthraquinon-sulfonate-(2)	432.8	0.15	6.72	-467	-0.218
		445.1	0.3			
		459.7	0.7			
	Safranin	512.0	9.3			
$\begin{array}{c} \text{C}_7\text{H}_{15}\text{.C} \begin{array}{l} \nearrow \text{N-N.C}_6\text{H}_5 \\ \searrow \text{N=N}^+ \end{array} \text{C}_6\text{H}_5 \\ \text{Cl}^- \end{array} \quad (15)$	anthraquinon-sulfonate-(2)	458.1	0.25	6.72	-478	-0.229
	Safranin	507.2	6.9			

In the case of triphenyl tetrazolium chloride, the reduction potential was also established for pH 5.89. The value is the same as that for pH 6.72. Thus the reduction potential in the tested area is not dependent on the acidity of the solvent, in contrast to the redox potential of numerous known stain indicators.

The new substances were subjected to an orientative test with corn seeds, with the cooperation of Dr. F. Moewus. No staining action on the viable areas of the germinal layer could be observed in the case of 5-methyl-2-phenyl-3-(o-carboxy-phenyl)-tetrazolium chloride. The corresponding p-carboxy compound produced a temporary pink coloration. Neither of these substances is suitable as an indicator of biological material, since the formazans produced by them are quite water soluble and thus may be further reduced to colorless compounds by the growing germinal tissue (the same is true for sodium dithionite solution at pH 7). The situation is more favorable in the case of 5-n-heptyl-2-phenyl-3-(o-carboxy-phenyl)-tetrazolium chloride; the resulting formazan is poorly soluble in water. This tetrazolium salt, suspended in phosphate buffer, produced colorations of the germinal layer, but these were considerably weaker than those evoked by triphenyl tetrazolium chloride and, surprisingly, limited to the vegetation points. The scutellum was not stained in almost every case.

The present investigation showed that the reduction potentials (deviation 1 mV/5 min.) of tetrazolium salts may be determined with precision. Tetrazolium compounds of distinctly differentiated reduction potentials may be produced by appropriate substitution. The basis is thereby established for work in biological investigations under aerobic conditions in the range of -0.080 to -0.230 volts (H_2 electrode) with indicators of calibrated potentials, the "leuco forms" of which are stable in atmospheric oxygen, in contrast to leucolactoflavin, indigo white and others.

Description of the tests.

The apparatus described by K. Wallenfels and W. Moehle (12) in the case of redox potential measurements is used for the determination of reduction potentials. It was equipped with an additional accessory 1, 2, 3, 4 (Fig. 4) (13). The electrometric titration is carried out in an electrode vessel 5 equipped with a number of ground faces for the introduction of electrodes, gas stream, burette and agar bridge to the calomel electrode. One of the ground faces is occupied by a dropping funnel 1, capable of holding two different substances. The two sections are closed off by stopcocks 2 and 4. The thin tubes attached thereto are for the passage of a gas stream. The nitrogen gas, passed through a KOH receiver and incandescent copper filings, enters through the titration vessel 5 (stopcock 3 is closed), through the slightly open stopcock 2 into the lower part of the dropping funnel 1 and permeates the fluid contained therein. Stopcock 4 is closed and bars the fluid in the upper portion. The nitrogen stream may also reduce the fluid located in the upper part via a tube which is inserted in the side of the vessel and which reaches well below the surface. The gas stream continues through the gas entry connection 6 and a connecting hose via the burette 7 into

the bubble counter and pressure receiver 8 or through the burette and an Erlenmayer flask 9 into the open. The titration vessel 5 is enclosed by a water sleeve and is held at 20°C by an ultrathermostat.

Process of measurement: 5×10^{-6} moles indicator substance in 30 cc buffer solution (pH 6.72 and 5.89; Phosphate buffer, pH 1.02; $n/10$ HCl) are placed in the titration vessel 5 and reduced within a few minutes by hydrogen after addition of one drop of 1% colloidal Pd solution. After a check of pH by means of a Pt-Mohr electrode the stream of hydrogen is replaced by a stream of nitrogen, meticulously freed of oxygen. After the hydrogen has been displaced from the apparatus (10 minutes), the lower portion of the dropping funnel is filled with 10 cc buffer solution (stopcock 2 closed; stopcock 3 open). Stopcock 3 is then closed and stopcock 2 slightly opened. Stopcock 4 is now closed and the upper portion of the dropping funnel is filled with 5×10^{-6} moles tetrazolium salt dissolved in 10 cc buffer solution. 15 cc of the solution of indicator substance required for the adjustment of the desired potential is placed in vessel 9. The system is prepared for measurement by passage for 1-2 hours.

The indicator solution contained in the flask 9 is compressed into the burette 7. A previously computed potential is adjusted by dropwise addition of reduced indicator from the titration vessel 5, containing the identical quantity in all tests. Measurements are carried out by means of 3 platinum electrodes against a calomel electrode according to the customary compensation method. Readings were precise within 0.2 mV, since a measuring wire 50 cm in length corresponded to 100 mV and a sensitive mirror galvanometer was used as a zero instrument.

First the buffer solution in the lower funnel space is led into the titration vessel by opening stopcocks 2 and 3; the potential deviation is noted. Generally a very small potential is obtained (less than 1 mV), depending on the indicator used. It is always measured, however, as a control. After 1 minute the potential value becomes constant; measurement is continued from the 5th to the 10th minute. Now the contents of the upper funnel space are added. The constantly flowing gas facilitates a thorough mixture of the tetrazolium salt solution with the indicator solution. If the indicator has a more negative potential than the tetrazolium salt, the start of a potential deviation may be observed already after $\frac{1}{2}$ minute. Its progression is measured after 1, 3, 5 and 15 minutes. The data depicted in Fig. 5 were obtained for triphenyl tetrazolium chloride.

Fig. 3 shows the 5 minute values. They differ from the measured data by the subtraction of a small blank value. This may be established in the case of potential values that produce a straight potential/time curve; in our present aggregate of curves, by extending the rectilinear curves toward time 0. The blank value is obtained at the intersection of the line with the ordinate. The corrected values, entered logarithmically in relation to the indicator potential, yield a straight line; its intersection with the line representing 1 mV indicates the desired reduction potential.

Indicators: The redox potential of the utilized indicators was measured and the progression of the titration curve fixed for pH 6.72, 1.02 and 5.89 (Table 3). It served as reference to the percentual share of oxidated and reduced forms for each indicator potential.

TABLE 3.

Indicator pH 1.02 (n/10-HCl)	E calomel elec. Volt	E norm. H ₂ elec. Volt	J ₁ mV	J ₂ mV
Anthrachinon-sulfonate-(2)(14)	-0.118	-0.131	14.1	14.7
Safranin (15)	-0.101	-0.148	14.0	13.7
Anthrachinon-disulfonate-(2.7) (14)	-0.073	-0.176	14.6	14.5
pH 6.72 (m/10 phosphate buffer)				
Safranin	-0.513	-0.269	13.5	13.0
Anthrachinon-sulfonate-(2)	-0.445	-0.196	15.0	14.0
Anthrachinon-disulfonate-(2.7)	-0.403	-0.154	16.5	16.3
Indigo disulfonate (15)	-0.335	-0.086	14.7	14.8
Indigo trisulfonate (15)	-0.303	-0.054	21.0	18.5
pH 5.89 (m/10 phosphate buffer)				
Anthrachinon-sulfonate-(2)	-0.397 (comp.)	-0.148	-	-

The data obtained from these curves must agree with those yielded by tests. This guarantees that none of the reduced indicator disappears during the passage of nitrogen due to oxygen traces, and that the added indicator solution is also completely free of oxygen.

The reduction potential is distinctly, if not considerably, dependent upon the concentrational ratio of acceptor to indicator. The example shown in Table 4 (acceptor: triphenyl tetrazolium chloride; indicator: indigo trisulfonate) shows this.

TABLE 4.

Ratio acceptor-indicator(*)	Adjusted indicator potential (calomel elec.)	mV of change 0-5 min.
2:1	-330.6 mV	0.7
1:1	-329.8 mV	0.9
1/2:1	-330.0 mV	1.3

(*) Amount of indicator: 5×10^{-6} mole.

The determination of the redox potential of compounds with reversible reactions: The same course may be taken here as in connection with irreversible systems. However, the union of indicator and acceptor is not marked by a reduction reaction, but by a constant potential value, i.e. the adjustment of a new equilibrium. Values obtained by this method are contained in Fig. 1 and 2. The rectilinear course is assured only if the indicator potential and the redox potential to be found are not more than 50 mV apart.

C-methyl-N-phenyl-N'-(p-carboxy-phenyl)-formazan: Was obtained quantitatively according to E. Bamberger and O. Billeter (4,10) in the manner described from the phenyl-hydrazine of acetaldehyde and the diazonium compound of p-amino-benzoic acid. From alcohol brick-red, shiny needles. Melting point 185°C (Berl).

$C_{15}H_{14}O_2N_4$ (282.1).

Computed: C 63.80 H 5.00 N 19.86.
Found: C 63.76, 63.94, H 5.23, 5.32, N 20.02, 19.96. (Micro-Dumas).

5-methyl-2-phenyl-3-(p-carboxy-phenyl)-tetrazolium chloride: 5 g C-methyl-N-phenyl-N'-(p-carboxy-phenyl)-formazan were dehydrated with 10 g lead tetraacetate in $CHCl_3$. After mixing with methyl-alcoholic hydrochloric acid, the desired tetrazolium salt was obtained in good yield. Crystals from methanol-acetic acid. Melting point 268°C (Berl, decomposition).

$C_{15}H_{13}O_2N_4Cl$ (316.6).

Computed: C 56.86 H 4.13 N 17.70
Found: C 56.96, 56.72, H 4.39, 4.28, N 17.79, 17.87. (Micro-Dumas).

C-methyl-N-phenyl-N'-(o-carboxy-phenyl)-formazan: The formazan was obtained in the same manner as the p-carboxy compound from dissociated o-aminobenzoic acid and acetaldehyde phenyl hydrazine. Crystallization from copious alcohol. Dark red needles. Melting point 200°C (Berl).

$C_{15}H_{14}O_2N_4$ (282.1).

Computed: C 63.80 H 5.00 N 19.86
Found: C 63.87, 63.76, H 5.35, 5.30, N 19.90 (Micro-Dumas).

5-methyl-2-phenyl-3-(o-carboxy-phenyl)-tetrazolium chloride: The dehydration of the appropriate formazan with lead tetraacetate led to tetrazolium salt in a smooth reaction. After treatment with hydrochloric acid and repeated precipitation it crystallized from alcohol-acetone after addition of ether. Melting point 242°C (Berl, decomposition).

$C_{15}H_{13}O_2N_4Cl$ (316.6)

Computed: C 56.86 H 4.13 N 17.70
Found: C 56.83, 56.82, H 4.96, 4.31, N 17.72, 17.56 (Micro-Dumas)

Equivalent weight 315.1

C-methyl-N-phenyl-N'-(p-oxy-phenyl)-formazan: From the diazonium salt of p-amino-phenol and acetaldehyde phenyl hydrazine. Crystals from alcohol. Melting point 148-149°C (Berl).

$C_{14}H_{14}ON_4$ (254.14).

Computed: C 66.11 H 5.55 N 22.05
Found: C 66.13, 66.39, H 5.62, 5.89, N 22.37 (Micro-Dumas).

5-methyl-2-phenyl-3-(p-oxy-phenyl)-tetrazolium chloride: From C-methyl-N-phenyl-N'-(p-oxy-phenyl)-formazan by dehydration with excessive lead tetraacetate in acetic acid. Needles from methanol ether. Melting point 261°C (Berl).

$C_{14}H_{13}ON_4Cl$ (288.6)

Computed: C 58.21, H 4.55, N 19.42, Cl 12.29.
Found: C 57.79, H 4.80, N 19.30, Cl 12.27.

C-n-heptyl-N-phenyl-N'-(o-carboxy-phenyl)-formazan: From 22 g of octylaldehyde phenyl hydrazine and 13.8 g of the diazonium compound of o-amino-benzoic acid. Yield 20 g of crude formazan. Brick-red needles from alcohol. Melting point 162°C (Berl).

$C_{21}H_{26}O_2N_4$ (366.2).

Computed: C 68.81, H 7.15, N 15.30.
Found: C 68.99, H 7.04, N 14.91.

5-n-heptyl-2-phenyl-3-(o-carboxy-phenyl)-tetrazolium chloride: 5 g of the analytically pure formazan described above were dehydrated with lead tetraacetate in $CHCl_3$. 3 g of crude tetrazolium salt were obtained after removal of the lead with methyl-alcoholic hydrochloric acid. Purification by twofold precipitation from alcohol with ether. Crystals from alcohol. Melting point 228-230°C (Berl, decomposition).

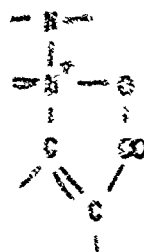
$C_{21}H_{25}O_2N_4Cl$ (400.68).

Computed: C 62.89 H 6.29 N 13.98.
Found: C 62.64, 62.61, H 6.56, 6.53, N 13.96.

NOTES.

(1) Comptes-rendus de l'Association Internationale d'Essais de Semences 1, 1 (1940); Ber. dtsh. bot. Ges. 57, 191 (1937); 60, 299, 434 (1942). — (2) R. Kuhn and D. Jerchel, Ber. 74, 949 (1941). — (3) see R. Wurmser in Handbuch der Enzymologie by Nord-Welddenhagen, Leipzig 1940, p. 298. — (4) R. Kuhn and D. Jerchel, Ber. 74, 941 (1941). — (5) Chem. Reviews 3, 1 (1926). — (6) Journ. Am. Chem. Soc. 52, 5204 (1930). — (7) He develops his method for the "critical oxidation potential" of unstable redox systems. — (8) Perhaps, following a thought of R. Kuhn,

the lowering of the potential is due to the possible formation of an hetero-polar ring; cf. *Naturwissenschaften*, 22, 618 (1932).



- (9) "Tetrazol" of the I.G. Farbenindustrie, AG, Leverkusen Plant. — (10) H. v. Pechmann and P. Stange, *Ber.* 27, 2920 (1894). — (11) E. Bamberger and O. Billster, *Helv. chim. Acta* 14, 231 (1931). — (12) *Ber.* 76, 933 (1943). — (13) see A.F. Cameron, *Journ. physic. Chem.* 42, 1217 (1938). — (14) Hays & Co, Berlin. — (15) E. Merck, Darmstadt.

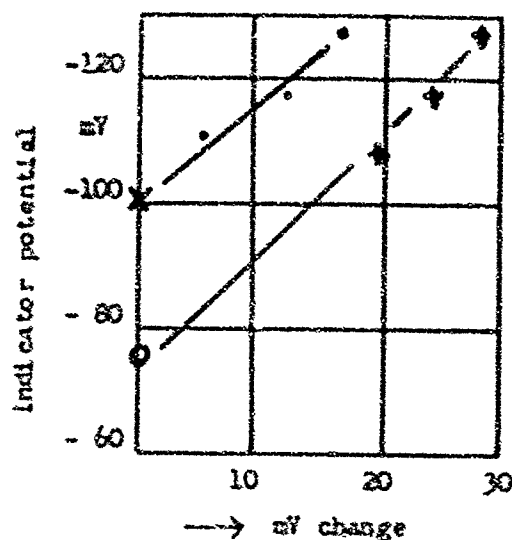


Fig. 1. pH 1.02. Indicator: anthrachinon-sulfonate-(2).
 — acceptor: anthrachinon-disulfonate-(2.7).
 . — . acceptor: safranin.
 O norm. pot. (calomel electrode) of anthrachinon-disulfonate-(2.7).
 X norm. pot. (calomel electrode) of safranin.

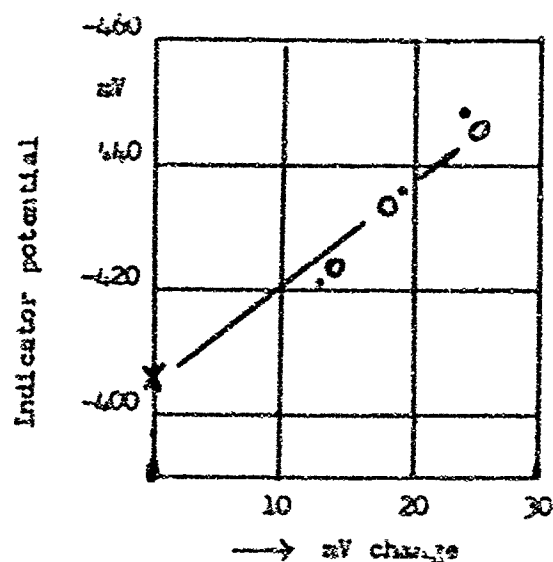


Fig. 2. pH 6.72. Indicator: anthrachinon-sulfonate-(2). acceptor: anthrachinon-disulfonate-(2.7).
 . — . Indicator: acceptor = 1:1
 O — O Indicator: acceptor = 1:2
 X norm. pot. (calomel electrode) of anthrachinon-disulfonate-(2.7).

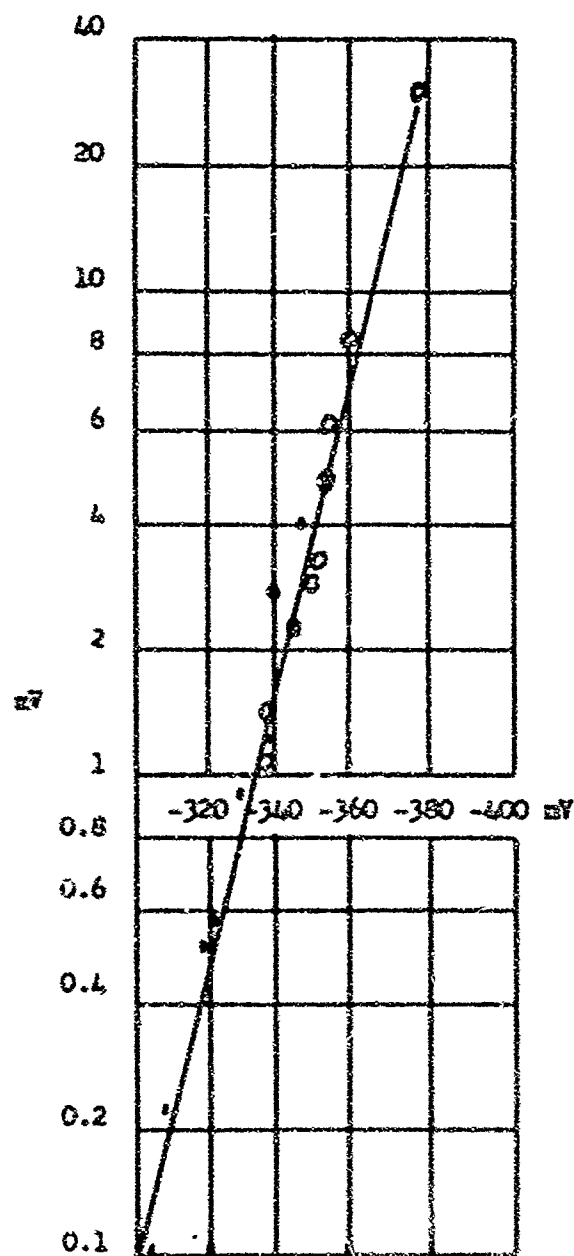
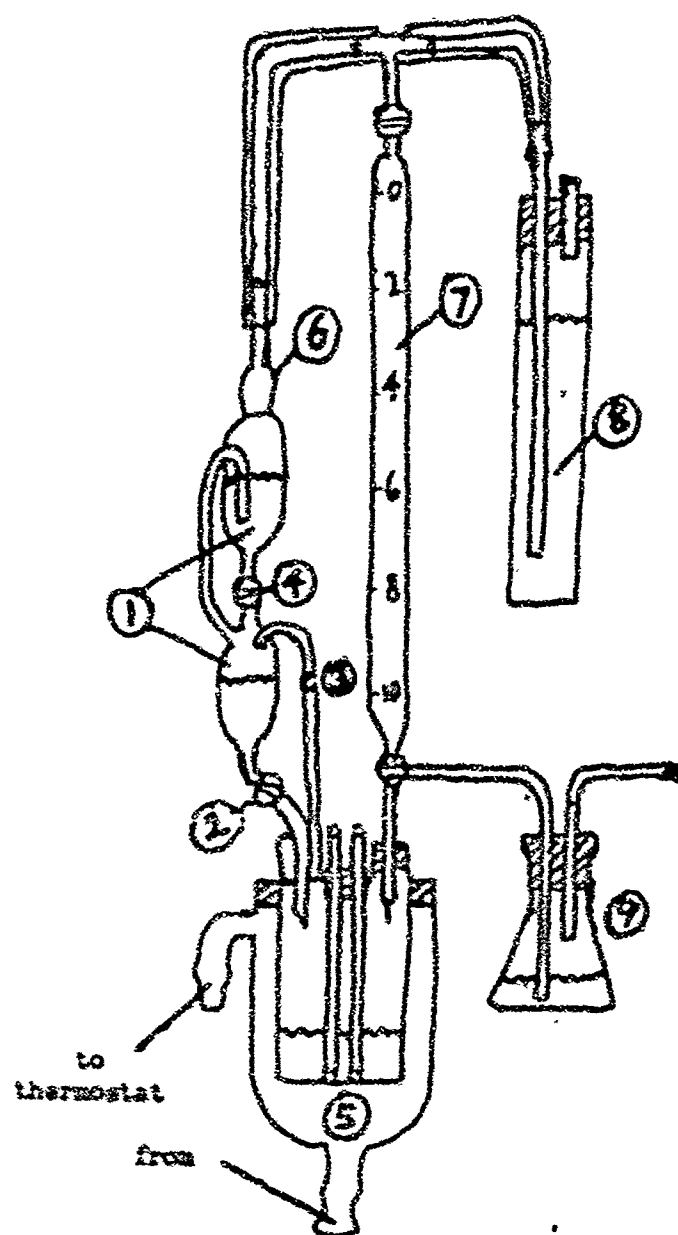


Fig. 3. Determination of reduction potential (change of 1 mV/0-5 min.) of triphenyl tetrazolium chloride. pH 6.72.
 . — . Indicator: indigo trisulfonate.
 ○ — ○ Indicator: indigo disulfonate.
 Abscissae: Indicator potential (calomel electrode).
 Ordinate: mV change/0-5 min. (logarithmic units)

Fig. 4. Apparatus for potential measurements.



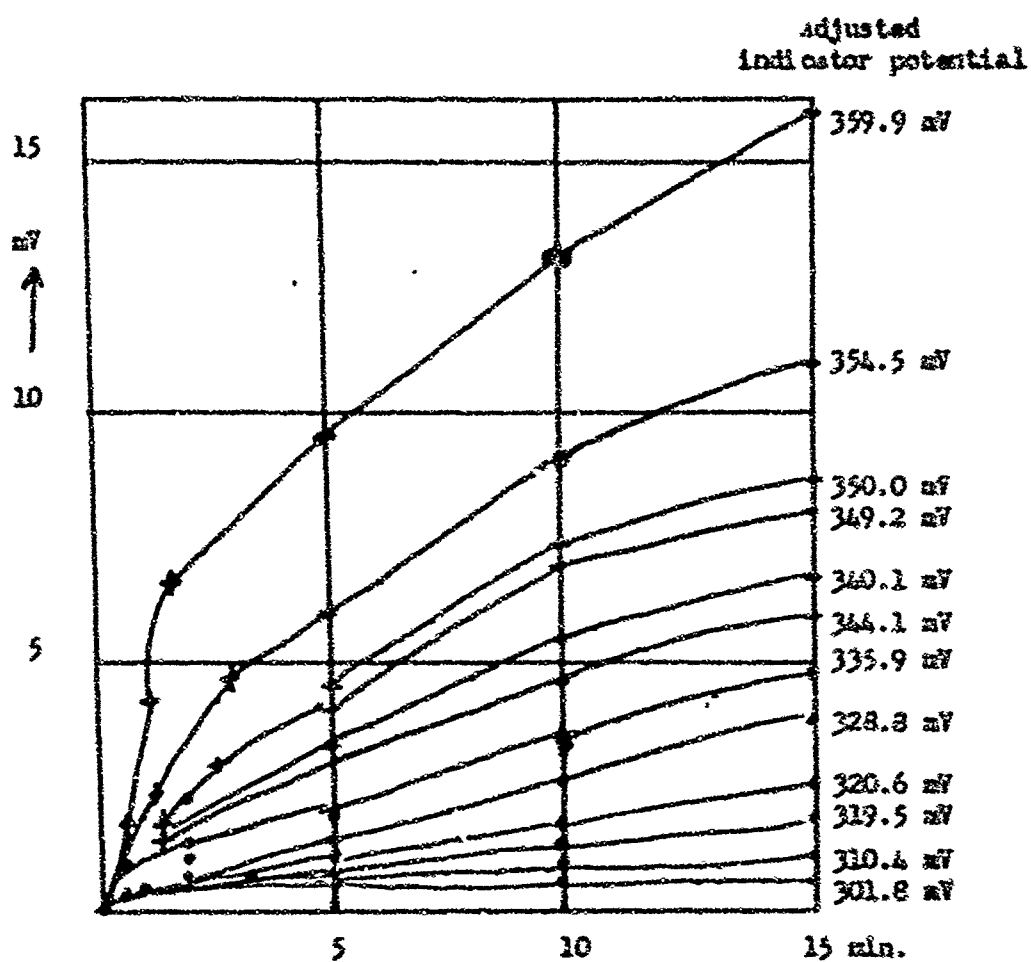


Fig. 5. pH 6.72. Progression of potential changes by means of triphenyl tetrazolium chloride.

—•—•— Indicator: Indigo trisulfonate.
 —x—x— Indicator: Indigo disulfonate.